

The bacterial gene *lfpA* influences the potent induction of calcitonin receptor and osteoclast-related genes in *Burkholderia pseudomallei*-induced TRAP-positive multinucleated giant cells

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Summary

Burkholderia pseudomallei is a facultative intracellular pathogen and the causative agent of melioidosis, a spectrum of potentially fatal diseases endemic in Northern Australia and South-East Asia. We demonstrate that *B. pseudomallei* rapidly modifies infected macrophage-like cells in a manner analogous to osteoclastogenesis. These alterations include multinucleation and the expression by infected cells of mRNA for factors required for osteoclastogenesis: the chemokines monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 gamma (MIP-1γ), 'regulated on activation normal T cell expressed and secreted' (RANTES) and the transcription factor 'nuclear factor of activated T-cells cytoplasmic 1' (NFATc1). An increase in expression of these factors was also observed after infection with *Burkholderia thailandensis*. Expression of genes for the osteoclast markers calcitonin receptor (CTR), cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) was also increased by *B. pseudomallei*-infected, but not by *B. thailandensis*-infected cells. The expression by *B. pseudomallei*-infected cells of these chemokine and osteoclast marker genes was remarkably similar to cells treated with RANKL, a stimulator of osteoclastogenesis.

Analysis of dentine resorption by *B. pseudomallei*-induced osteoclast-like cells revealed that demineralization may occur but that authentic excavation does not take place under the tested conditions. Furthermore, we identified and characterized *lfpA* (for lactonase family protein A) in *B. pseudomallei*, which shares significant sequence similarity with the eukaryotic protein 'regucalcin', also known as 'senescence marker protein-30' (SMP-30). *LfpA* orthologues are widespread in prokaryotes and are well conserved, but are phylogenetically distinct from eukaryotic regucalcin orthologues. We demonstrate that *lfpA* mRNA expression is dramatically increased in association with macrophage-like cells. Mutation of *lfpA* significantly reduced expression of the tested host genes, relative to the response to wild-type *B. pseudomallei*. We also show that *lfpA* is required for optimal virulence *in vivo*.

Introduction

Burkholderia pseudomallei is a Gram-negative bacterium that causes melioidosis, a collective term describing a broad spectrum of diseases afflicting animals and humans. The major endemic foci of melioidosis are Northern Australia and South-East Asia. *B. pseudomallei* can be readily isolated from moist soil and still water in endemic regions and infection is acquired by inhalation, percutaneous inoculation, or ingestion (Currie *et al.*, 2000a; Cheng and Currie, 2005). Melioidosis has a spectrum of clinical presentations that can mimic many other infections and can affect most organs (Leelarasamee and Bovornkitti, 1989). Severe melioidosis results in approximately 50% mortality in Thailand (White, 2003) and the disease has 19% mortality in Australia (Currie *et al.*, 2000b). *B. pseudomallei* is recognized as a potential bio-warfare agent (Bossi *et al.*, 2004) and no vaccine currently exists against melioidosis.

Burkholderia pseudomallei is a facultative intracellular pathogen; it can invade, survive and replicate inside phagocytic and non-phagocytic cells (Pruksachartvuthi *et al.*, 1990; Harley *et al.*, 1994; Jones *et al.*, 1996) and *Acanthamoeba* (Inglis *et al.*, 2000). Following entry,

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14. ABSTRACT

Burkholderia pseudomallei is a facultative intracellular pathogen and the causative agent of melioidosis, a spectrum of potentially fatal diseases endemic in Northern Australia and South-East Asia. We demonstrate that **B. pseudomallei** rapidly modifies infected macrophage-like cells in a manner analagous to osteoclastogenesis. These alterations include multinucleation and the expression by infected cells of mRNA for factors required for osteoclastogenesis: the chemokines monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 1 gamma (MIP-1gamma), 'regulated on activation normal T cell expressed and secreted' (RANTES) and the transcription factor 'nuclear factor of activated T-cells cytoplasmic 1' (NFATc1). An increase in expression of these factors was also observed after infection with **Burkholderia thailandensis**. Expression of genes for the osteoclast markers calcitonin receptor (CTR), cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP) was also increased by **B. pseudomallei**-infected, but not by **B. thailandensis**-infected cells. The expression by **B. pseudomallei**-infected cells of these chemokine and osteoclast marker genes was remarkably similar to cells treated with RANKL, a stimulator of osteoclastogenesis. Analysis of dentine resorption by **B. pseudomallei**-induced osteoclast-like cells revealed that demineralization may occur but that authentic excavation does not take place under the tested conditions. Furthermore, we identified and characterized lfpA (for lactonase family protein A) in **B. pseudomallei**, which shares significant sequence similarity with the eukaryotic protein 'regucalcin', also known as 'senescence marker protein-30' (SMP-30). LfpA orthologues are widespread in prokaryotes and are well conserved, but are phylogenetically distinct from eukaryotic regucalcin orthologues. We demonstrate that lfpA mRNA expression is dramatically increased in association with macrophage-like cells. Mutation of lfpA significantly reduced expression of the tested host genes, relative to the response to wild-type **B. pseudomallei**. We also show that lfpA is required for optimal virulence in vivo.

15. SUBJECT TERMS

Burkholderia pseudomallei, multinucleated giant cells, osteoclastogenesis, calcitonin, chemokines

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B. pseudomallei has been observed in the cytoplasm following destruction of the 'phagosome' (Harley *et al.*, 1998a), indicating that *B. pseudomallei* is able to lyse intracellular vesicles and proliferate in the cytoplasm. The *bsa* locus is essential for escape from intracellular vesicles and for intracellular replication (Stevens *et al.*, 2002). The ability to survive and proliferate inside eukaryotic cells is likely to be important for the latency, recrudescence, granulomas and difficulties in treatment associated with melioidosis (see reviews by Currie *et al.*, 2000c; White, 2003).

Burkholderia pseudomallei-infected mononuclear macrophage-like cells develop into multinucleated giant cells (MNGCs) in culture (Harley *et al.*, 1998b), a result of cell fusion (Kespichayawattana *et al.*, 2000). Previous studies have also reported the observation of MNGCs in melioidosis patients (Sirikulchayanonta and Subhadrabandhu, 1994; Wong *et al.*, 1995). Multinucleation arises *in vitro* by the fusion of mononuclear macrophage-like cells into MNGCs (Kespichayawattana *et al.*, 2000). 'Giant' cells containing many intracellular bacteria have also been observed in post-mortem samples from human melioidosis patients (Wong *et al.*, 1995) and from biopsies from *B. pseudomallei*-infected bone (Sirikulchayanonta and Subhadrabandhu, 1994), indicating that cell entry, intracellular survival and proliferation, and MNGC development occur *in vivo*. That these MNGCs are not activated macrophages is suggested by the observation that *B. pseudomallei*-infected macrophage-like cells fail to produce inducible nitric oxide synthase (iNOS), in contrast to *Escherichia coli*- and *Salmonella enterica* serovar Typhi-infected cells (Utai-incharoen *et al.*, 2001).

The relevance of MNGCs to virulence and pathogenesis of *B. pseudomallei* is currently unclear; however, it seems likely that changes in gene expression in macrophages leading to MNGC formation may be involved either in subversion of the immune system or in bone infections that are known to be induced by *B. pseudomallei*. Furthermore, very little is known about how bacterially induced cell fusion occurs and what bacterial and host cell molecules are involved. To date, only the type III secretion effector protein BipB, and the global regulatory factor RpoS, have been implicated in *B. pseudomallei*-induced MNGC formation, as mutations in either gene severely attenuate MNGC formation (Suparak *et al.*, 2005; Utai-incharoen *et al.*, 2006).

Osteoclasts are also MNGCs derived from the fusion of mononuclear cells of the monocyte/macrophage lineage (Teitelbaum, 2000). They are characterized by the expression of functional osteoclast markers calcitonin receptor (CTR), cathepsin K (CTSK) and tartrate-resistant acid phosphatase (TRAP; Nicholson *et al.*, 1986; Gelb *et al.*, 1996; Gowen *et al.*, 1999; Igarashi *et al.*, 2002). Precursor

cells can be induced to form genuine osteoclasts *in vitro* by addition of 'receptor activator of nuclear factor- κ B ligand' (RANKL) in the absence of colocated stromal cells or osteoblasts (Hsu *et al.*, 1999). In addition, a number of chemokines and a transcription factor have been implicated in multinucleated cell formation and osteoclastogenesis. These include the chemokines monocyte chemoattractant protein 1 (MCP-1), macrophage inflammatory protein 1 gamma (MIP-1 γ), 'regulated on activation normal T cell expressed and secreted' (RANTES) and the transcription factor 'nuclear factor of activated T-cells cytoplasmic 1' (NFATc1) (Cappellen *et al.*, 2002; Ishida *et al.*, 2002; Lean *et al.*, 2002; Kyriakides *et al.*, 2004; Day *et al.*, 2005).

Osteoclast-like cells have been observed following bacterial treatment, albeit after long incubation times (Nair *et al.*, 1996; Abu-Amer *et al.*, 1997). As *B. pseudomallei* and *Burkholderia thailandensis* induce MNGC formation, and osteoclasts are MNGCs, we investigated whether any similarity exists between osteoclasts and *B. pseudomallei*-induced or *B. thailandensis*-induced MNGCs. We also identified a bacterial gene, *lfpA*, that is present in *B. pseudomallei* and absent from *B. thailandensis*, and whose product modulates the host cell response to *B. pseudomallei* *in vitro* and virulence *in vivo*.

Results

Burkholderia pseudomallei potently and rapidly induces osteoclast-like cell formation

We first confirmed that *B. pseudomallei* could induce MNGC formation from RAW264.7 cells. After 12 h of infection, cells infected with *B. pseudomallei* or *B. thailandensis* were multinucleate and indistinguishable by microscopy (Fig. 1). Bacteria residing within MNGCs were readily observed, as were actin tails associated with bacteria. Uninfected cells remained mononuclear. In contrast, RAW264.7 cells treated with RANKL for 12 h remained mononuclear. Expression by the host cells of chemokines, NFATc1, and osteoclast markers were analysed following infection or RANKL treatment, and compared with untreated cells (Fig. 2). *B. pseudomallei*-infected RAW264.7 cells produce significantly more mRNA than uninfected cells for MCP-1, MIP-1 γ , RANTES and NFATc1 ($P < 0.0001$). When compared with RAW264.7 cells exposed to RANKL for 12 h, expression by *B. pseudomallei*-infected cells was similar for RANTES ($P = 0.89$), and MCP-1 ($P = 0.07$), whereas infected cells produced more MIP-1 γ than 12 h RANKL-treated cells ($P = 0.0004$). Slightly more mRNA for NFATc1 ($P = 0.02$) was produced by 12 h RANKL-treated cells.

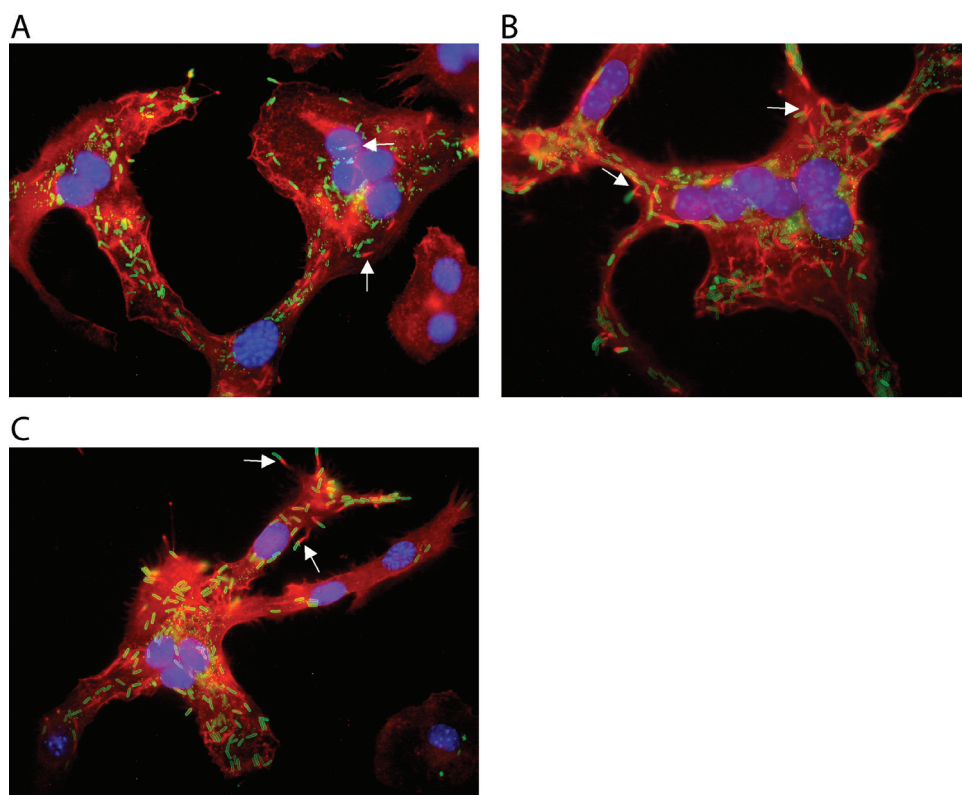


Fig. 1. False colour images of bacterially induced MNGC formation, and intracellular bacterial proliferation. RAW264.7 cells were infected with (A) *B. pseudomallei* strain 08, (B) *B. thailandensis* E264 or (C) *B. pseudomallei* strain JAB136 ($\Delta lfpA$). Bacteria are labelled green, nuclei are blue and actin is red. White arrows indicate bacteria with associated actin tails.

Bacterially infected cells were then assessed for expression of osteoclast markers (Fig. 2B). CTR, CTSK and TRAP were all expressed significantly more ($P < 0.0001$) by *B. pseudomallei*-infected than uninfected cells. The level of CTSK and TRAP expression by *B. pseudomallei*-infected cells was similar to 12 h RANKL-treated cells ($P = 0.16$ and 0.82 respectively). Notably, CTR expression levels were 365-fold greater in *B. pseudomallei*-infected cells than in 12 h RANKL-treated cells ($P < 0.0001$), and 51-fold more than in 5 day RANKL-treated cells ($P < 0.0001$).

Collectively, these data indicate that RAW264.7 cells are potently and rapidly modified during *B. pseudomallei* infection. The expression of chemokines, NFATc1 and functional markers of osteoclasts by *B. pseudomallei*-infected cells strongly resembles that of RAW264.7 cells treated with RANKL for 12 h.

Burkholderia thailandensis does not induce the same host response as *B. pseudomallei*

Burkholderia thailandensis is closely related to *B. pseudomallei* but is widely considered to be avirulent

and has reduced virulence in the hamster model of infection (Brett *et al.*, 1997). In line with a previous report, we observed MNGCs following *B. thailandensis* infection of RAW264.7 cells (Harley *et al.*, 1998b). *B. thailandensis*-infected cells expressed significantly more mRNA for the chemokines MCP-1, MIP-1 γ and RANTES than uninfected cells ($P < 0.0001$). The high-level induction of chemokines did not correlate with NFATc1 expression, which was only 2.4-fold higher in *B. thailandensis*-infected cells compared with uninfected cells ($P = 0.02$). Compared with *B. pseudomallei*-infected cells, *B. thailandensis*-infected cells expressed significantly less MCP-1 ($P = 0.0003$), and NFATc1 ($P = 0.0003$), but similar levels of MIP-1 γ ($P = 0.017$) and RANTES ($P = 0.07$) (Fig. 2A).

The cellular response to infection was strikingly different with respect to the osteoclast markers CTR, CTSK and TRAP: these genes were expressed at significantly lower levels in *B. thailandensis*-infected cells relative to *B. pseudomallei*-infected cells ($P < 0.0001$). In fact, osteoclast marker gene expression by *B. thailandensis*-infected cells was the same as, or less than uninfected cells (Fig. 2B).

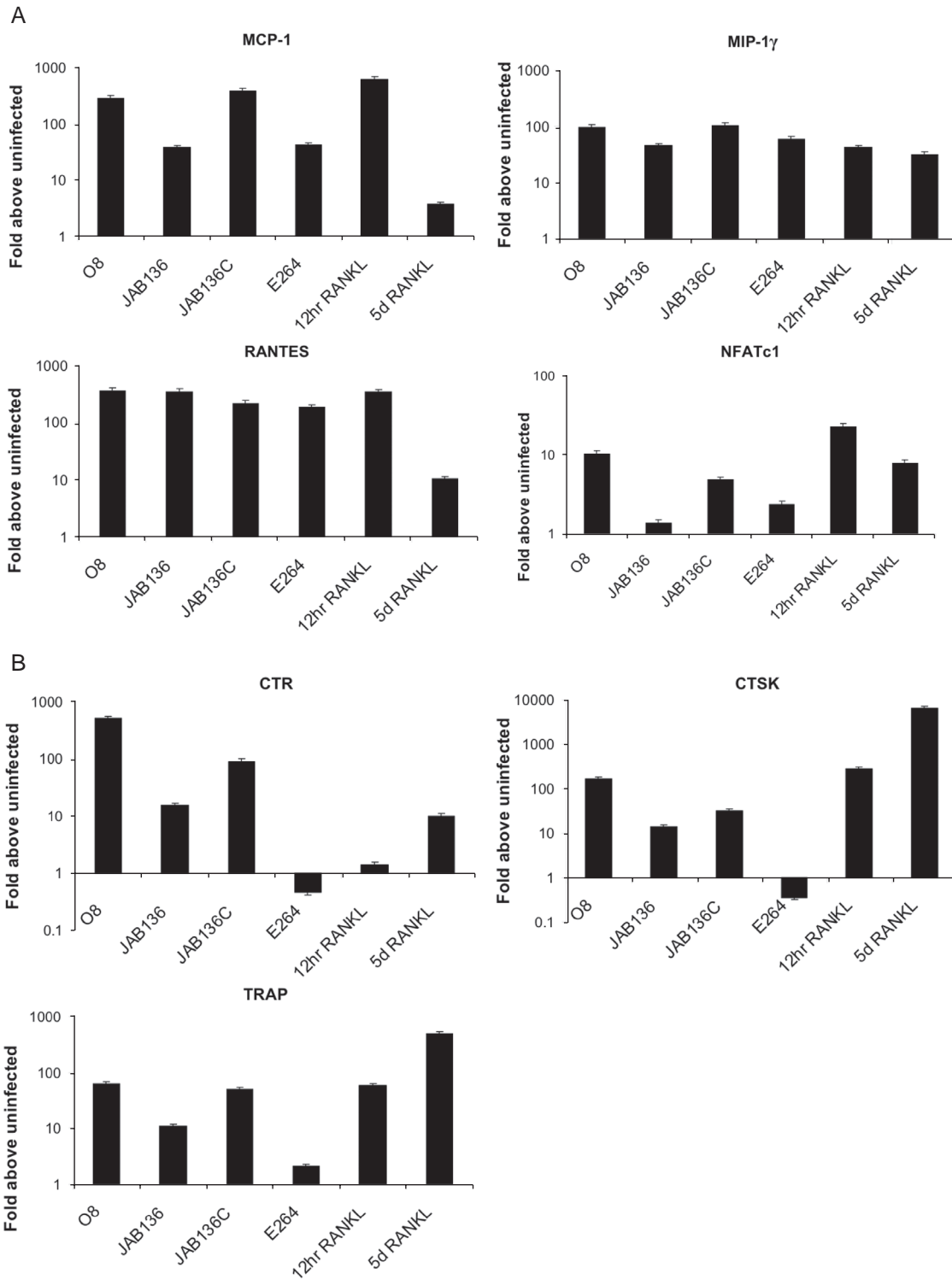


Fig. 2. Expression of (A) chemokines, NFATc1 and (B) osteoclast markers by infected RAW264.7 cells. mRNA for osteoclast markers, chemokines, or NFATc1 per 18S mRNA was obtained following 12 h RANKL treatment, or 5 day RANKL treatment of RAW264.7 cells, and following infection with *B. pseudomallei* O8 (wt), JAB136 ($\Delta lfpA$), JAB136C (complemented $\Delta lfpA$), or *B. thailandensis* E264. The mean \pm SEM per cent of mRNA copies per 18S rRNA was divided by mRNA copies per 18S rRNA from uninfected RAW264.7 cells 12 h after plating (untreated).

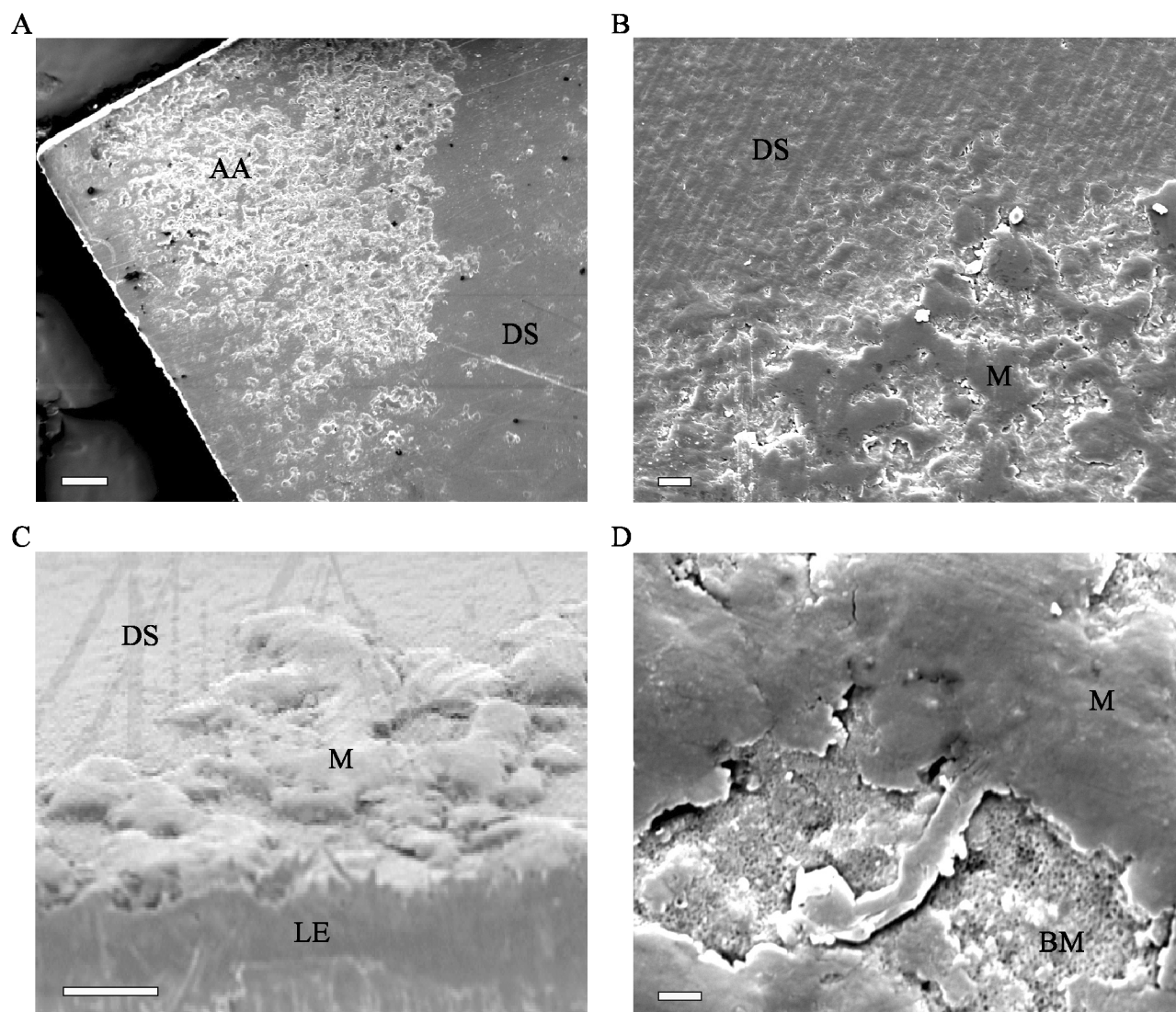


Fig. 3. Effects of *B. pseudomallei*-infected RAW264.7 cells on dentine. Images show electron micrographs of dentine slices following coculture with *B. pseudomallei*-infected RAW264.7 cells for 24 h.

A. 70× magnification illustrating a large 'affected' area (AA) in the top left-hand corner of the dentine slice, resembling resorptive lacunae. The unaffected dentine surface (DS) is indicated. Bar represents 250 µm.

B. 300× magnification illustrating no resorptive lacunae; rather, mounds (M) rising from the dentine surface (DS). Striations in the dentine surface are from a dentine saw, allowing clear identification of the dentine surface. Bar represents 10 µm.

C. 1300× magnification of a cross-section of dentine cut through an 'affected' area. The leading edge, cut with a scalpel, is indicated (LE). The dentine surface can be seen (DS) as can mounds (M) rising from the dentine surface. No authentic resorptive lacunae can be seen. Bar represents 50 µm.

D. 10 000× magnification of an 'affected' area, illustrating mounds (M) and regions below the mounds, resembling demineralized matrix (BM). Bar represents 2 µm.

Are B. pseudomallei-infected cells authentic osteoclasts?

We examined whether *B. pseudomallei*-infected cells could resorb dentine. At low magnification (70×), the surfaces of dentine appear to have substantial resorption (Fig. 3A). However, on closer inspection (1300×) the 'affected' regions of dentine do not constitute resorptive lacunae; rather, they appear as mounds rising from the

surface of the dentine, and no authentic resorptive lacunae were identified (Fig. 3B and C). Raised areas of mineral on dentine surface appears to be a previously unreported phenomenon. Similar structures are absent both from RAW264.7 cell cultures on dentine without RANKL and from osteoclast cultures on dentine. The chemical nature of this phenomenon is under further investigation. The same result was observed over triplicate experiments. Importantly, however, very high magni-

Table 1. Ten most similar prokaryotic and eukaryotic homologues of *B. pseudomallei* LfpA.

Organism	Homologue name	GenBank accession	% Similarity (% identity)	Alignment length ^a
Prokaryotes				
<i>Mesorhizobium loti</i>	Regucalcin homologue	NP_107711	52 (37)	305
<i>Pseudomonas fluorescens</i>	SMP-30	YP_349871	52 (36)	300
<i>Pseudomonas syringae</i>	SMP-30	AAY37409	51 (37)	298
<i>Nostoc punctiforme</i>	Gluconolactonase	ZP_00110023	49 (31)	305
<i>Sinorhizobium meliloti</i>	Putative regucalcin	NP_435274	48 (33)	316
<i>Burkholderia fungorum</i>	Gluconolactonase	ZP_00283124	48 (32)	306
<i>Coxiella burnetti</i>	Hypothetical protein	NP_820769	48 (31)	302
<i>Bacillus clausii</i>	Gluconolactonase	YP_176297	48 (30)	305
<i>Burkholderia cepacia</i>	Gluconolactonase	ZP_00212210	46 (35)	315
<i>Microbulbifer degradens</i>	Gluconolactonase	ZP_00317728	45 (31)	302
Eukaryotes				
<i>Xenopus laevis</i>	Regucalcin	BAA90694	48 (29)	305
<i>Mus musculus</i>	Regucalcin	BAA13046	48 (27)	305
<i>Oryctolagus caniculus</i>	Regucalcin	BAA88079	47 (29)	305
<i>Aspergillus fumigatus</i>	Regucalcin homologue	EAL89928	47 (28)	305
<i>Gallus gallus</i>	Regucalcin	BAA90693	46 (29)	305
<i>Homo sapiens</i>	Regucalcin	NP_004674	46 (27)	305
<i>Rattus norvegicus</i>	Regucalcin	NP_113734	46 (26)	305
<i>Bos taurus</i>	Regucalcin	BAA88080	45 (27)	305
<i>Fugu rubripes</i>	SINFRUP00000140211 ^b	Not applicable	42 (27)	304
<i>Anopheles gambiae</i>	ENSANGP00000010332	EAA12326	42 (27)	312

a. Number of amino acids in the full-length protein alignment, including gaps.

b. Ensembl Peptide ID.

fication (10 000×) of affected areas revealed regions of dentine resembling demineralized matrix (Fig. 3D).

Taken together, these data indicate that *B. pseudomallei*-infected osteoclast-like cells are not authentic osteoclasts, as typical resorptive lacunae were not observed; however, apparent demineralization of dentine without excavation of the dentine matrix occurred in some areas following 24 h of coculture with *B. pseudomallei*-infected RAW264.7 cells.

Identification and sequence analysis of *lfpA* in *B. pseudomallei*

Because *B. pseudomallei* and *B. thailandensis* differ markedly in the ability to induce osteoclast-related genes in the host cell RAW264.7, we reasoned that there must exist in *B. pseudomallei* genes that control this trait and that such genes would be absent from *B. thailandensis*. Furthermore, such genes may be induced in the presence of the host cell and contribute to the differential virulence of *B. thailandensis* and *B. pseudomallei*. Genes previously identified as *B. pseudomallei*-specific by subtractive hybridization between *B. pseudomallei* strain 08 and *B. thailandensis* strain E264 were examined (Brown and Beacham, 2000). One gene fulfilling the criteria was represented by a *B. pseudomallei*-specific clone (pSC-A36) that contains the *B. pseudomallei* K96243 gene BPSS2074, annotated as 'senescence marker protein-30 (SMP-30) family protein' (http://www.sanger.ac.uk/Projects/B_pseudomallei/). BPSS2074 is located within a

61.8 kb genomic island on chromosome two (GI16), and contains 59.5% GC, compared with the chromosome two mean of 68.5% GC (Holden *et al.*, 2004). BLASTP (Altschul *et al.*, 1997) searches using the predicted full-length primary amino acid sequence identified orthologues that are gluconolactonases or putative gluconolactonases (summarized in Table 1). The *B. pseudomallei* protein was designated LfpA (for lactonase family protein A) and BPSS2074 was designated *lfpA*. *lfpA* is a 891 bp gene that encodes a predicted protein of 296 amino acids with a calculated molecular mass of 32 260 Da and a projected pI of 4.90. The *lfpA* reading frame is in the opposite orientation relative to adjacent genes, suggesting that it is not part of an operon. A signal peptide was not predicted for LfpA by SIGNALP 3.0. However, a putative prokaryotic membrane lipoprotein lipid attachment site was identified in the N-terminus of LfpA by the ScanProsite server, indicating that LfpA may be a post-translationally processed membrane-associated lipoprotein.

Southern blot analysis identified *lfpA* in five of eight *B. pseudomallei* strains (see Table 2) but not in *Burkholderia cepacia* strain ATCC17765 (clinical isolate) or *B. thailandensis* strain E264 (results not shown). LfpA orthologues were also identified in *Burkholderia fungorum* LB400 (GenBank Accession ZP_00283124) and *B. cepacia* R18194 (ZP_00212210) but were not identified in the genome sequences of *Burkholderia mallei* ATCC23344 or *B. thailandensis* E264. There was no correlation between the presence of *lfpA* and clinical or environmental source of the *B. pseudomallei* strains.

Table 2. Bacterial strains and plasmids used in this study.

Species/strain/plasmid	Genotype/source	Reference
Bacterial strains		
<i>Burkholderia pseudomallei</i>		<i>lfpA</i> ^a
08	wt, clinical isolate Gm ^r Sm ^r Cm ^s Tc ^s	Y
JAB136.1x	08 derivative; <i>lfpA</i> ::pJAB136 (Δ <i>lfpA</i> [nt92–789], <i>cat</i> , <i>sacB</i> , <i>oriT</i> , <i>oriR6K</i>); Gm ^r Sm ^r Cm ^r Tc ^s	Brown and Beacham (2000) This study
JAB136	08 derivative; Δ <i>lfpA</i> (nt92–789); Gm ^r Sm ^r Cm ^s Tc ^s	This study
JAB136C	08 derivative; JAB136 harbouring plasmid pJAB136C; Gm ^r Sm ^r Cm ^s Tc ^r	This study
DD503	1026b derivative; Δ (<i>amrA-oprA</i>) <i>rpsL</i> ; Sm ^r Gm ^s Km ^s	Y
RU136	DD503 derivative; <i>lfpA</i> ::pRU136; Sm ^r Gm ^r Km ^s	Y
K96243	wt, clinical isolate	Y
06	wt, environmental isolate	Y
GF1	wt, environmental isolate	Y
GF2	wt, environmental isolate	Y
GCH	wt, clinical isolate	N
THP375	wt, environmental isolate	N
THP385	wt, clinical isolate	N
<i>Burkholderia thailandensis</i>		
E264	wt, environmental isolate;	N
<i>Escherichia coli</i>		
DH5 α	<i>supE44</i> Δ <i>lacU169</i> (ϕ 80 <i>lacZ</i> Δ M15) <i>hsdR17</i> <i>recA1</i> <i>endA1</i> <i>gyrA96</i> <i>thi-1</i> <i>reiA1</i>	Brett <i>et al.</i> (1997)
S17.1 (λ pir)	RP4-2-Tc::Mu Km::Tn7 Tp Sm(λ pir) <i>phoA20</i> <i>thi-1</i> <i>rpsE</i> <i>rpoB</i> <i>araE</i> (Am) <i>recA1</i>	Brown (1991)
SM10	RP4 <i>tra</i> genes, Km ^r	Penfold and Pemberton (1992)
JABEC136	S17.1 (λ pir) containing pJAB136	Simon <i>et al.</i> (1983)
JABEC136C	S17.1 (λ pir) containing pJAB136C	This study
RUEC136	SM10 containing pRU136	This study
Plasmids		
pGEM-T Easy	TA cloning vector; <i>ori</i> , <i>oriF1</i> , <i>lacZ</i> ; Ap ^r	Promega Corporation
pDM4	<i>sacBR</i> , <i>oriT</i> , <i>oriR6K</i> ; Cm ^r	Milton <i>et al.</i> (1996)
pBBR1MCS-3	<i>ori</i> , <i>lacZ</i> ; Tc ^r	Kovach <i>et al.</i> (1995)
pCR2.1-TOPO	TA cloning vector; Km ^r Ap ^r	Invitrogen Corporation
pGSV3	Mobilizable suicide vector; Gm ^r	DeShazer <i>et al.</i> (2001)
pJAB136easy	pGEM-T Easy with 1764 bp fragment, representing Δ <i>lfpA</i> (nt92–789)	This study
pJAB136	pDM4 with ~1790 bp <i>SpeI</i> - <i>SphI</i> fragment from pJAB136easy, representing Δ <i>lfpA</i> (nt92–789)	This study
pJAB136Ceasy	pGEM-T Easy with 1072 bp fragment, representing wild-type <i>lfpA</i> and putative promoter region	This study
pJAB136C	pBBR1MCS-3 with 1068 bp <i>XbaI</i> fragment from pJAB136Ceasy, representing wild-type <i>lfpA</i> and putative promoter region	This study
pRU136TOPO	pCR2.1-TOPO with 803 bp amplicon internal to <i>lfpA</i> from DD503	This study
pRU136	pGSV3 with ~810 bp <i>EcoRI</i> fragment from pRU136TOPO	This study

a. Presence of *lfpA* in *Burkholderia* wt strains as determined by Southern blot or PCR.

b. D. Alfredson, Gold Coast Hospital, Queensland, Australia. Present address: St. John of God Hospital, Geelong, Victoria, Australia. Ap, ampicillin; Cm, chloramphenicol; Gm, gentamicin; Km, kanamycin; Sm, streptomycin; Tc, tetracycline; s, sensitive; r, resistant.

LfpA is expressed in association with macrophage-like cells

Expression of *lfpA* mRNA in *B. pseudomallei* 08 was investigated in the presence and absence of RAW264.7 cells to determine whether the host–bacterium interaction affects expression. After growth for 3 h in the absence of RAW264.7 cells, 8.7×10^{-10} ($\pm 3.5 \times 10^{-10}$) *lfpA* copies per 16S rRNA were detected. However, after growth for 3 h in the presence of RAW264.7 cells, 8.3×10^{-3} ($\pm 9.7 \times 10^{-4}$) *lfpA* copies per 16S rRNA were detected. This represents a 9.6×10^6 -fold increase in

expression ($P < 0.0001$), indicating that *lfpA* expression is potentially upregulated in contact with RAW264.7 cells or in medium surrounding such cells. When *lfpA* was reintroduced into JAB136 on a plasmid, this complemented strain JAB136C expressed 1.5×10^{-3} ($\pm 7.1 \times 10^{-4}$) *lfpA* copies per 16S rRNA in contact with RAW264.7 cells (18.5% of the wild-type strain, $P = 0.04$).

LfpA modulates host cell expression changes

Because *lfpA* is upregulated in association with RAW264.7 cells, we investigated whether this gene

Table 3. mRNA expression relative to uninfected cells.

Infecting strain/treatment	Osteoclast markers			Chemokines and NFATc1			
	CTR	CTSK	TRAP	NFATc1	MIP-1 γ	MCP-1	RANTES
<i>B. pseudomallei</i> 08 (WT)	512	169	63	10	97	289	362
JAB136 (<i>lfpA</i> deletion)	15	14	11	1.4	45	38	354
JAB136C (complemented)	91	33	50	4.8	104	388	218
<i>B. thailandensis</i> E264	0.46	0.35	2.1	2.4	60	42	185
RANKL 12 h	1.4	287	58	23	42	630	346
RANKL 5 day	10	6654	489	8	32	3.7	10

affects the osteoclast-like pattern of gene expression observed during *B. pseudomallei* infection by comparing host cell gene expression following infection with wild-type (strain 08), JAB136 ($\Delta lfpA$ strain) or JAB136C (*trans*-complemented $\Delta lfpA$ strain). While MNGCs were observed 12 h post infection with either *B. pseudomallei* 08 or the $\Delta lfpA$ strain JAB136 (Fig. 1A and C), JAB136-infected cells produced significantly less MCP-1 ($P = 0.002$), MIP-1 γ ($P = 0.0008$) and NFATc1 ($P < 0.0001$) mRNA than 08-infected cells (Fig. 2A). Similarly, the osteoclast markers CTR, CTSK and TRAP were expressed significantly less ($P < 0.0001$) by JAB136-infected cells compared with 08-infected cells (Fig. 2B). The defect in MCP-1, MIP-1 γ and TRAP mRNA expression was fully restored when *lfpA* was reintroduced into JAB136 on a plasmid, and partially restored for NFATc1, CTR and CTSK (see Fig. 2A and B, Table 3). These data indicate that *lfpA* is required for optimal expression of all genes tested except RANTES in *B. pseudomallei*-infected RAW264.7 cells, compared with uninfected cells, and that the reduced expression of *lfpA* in the complemented strain is reflected by reduced expression of NFATc1, CTR and CTSK.

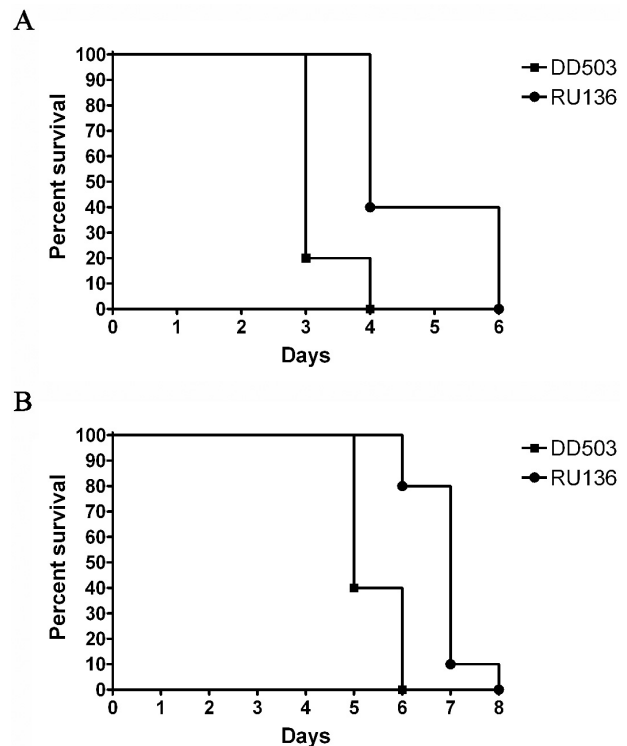
LfpA is not required for intracellular proliferation

To determine whether the host cell responses observed following JAB136 infection relate to intracellular survival and proliferation, the ability of JAB136 to proliferate in RAW-264.7 cells was compared with the parental strain, *B. pseudomallei* strain 08. Strains 08 and JAB136 replicated 258 ± 47 -fold and 268 ± 23 -fold, respectively, after 8 h. These similar rates of replication indicate that *lfpA* is not required for intracellular replication ($P = 0.77$). This result also implies that the difference in the response of RAW264.7 cells to infection by JAB136 or 08 is not the result of reduced intracellular JAB136 bacteria.

LfpA is required for optimal virulence in vivo

As *lfpA* is required for optimal changes in host cell expression *in vitro*, we investigated whether this gene has a role *in vivo*. In the Syrian hamster model of infection (Brett

et al., 1997), the relative LD₅₀ for strain RU136 (*lfpA* mutant) was 95 cfu compared with < 21 cfu for strain DD503 (parental strain), representing a > 4.5 -fold increase in LD₅₀ for the mutant strain. Median survival was 25% greater for the RU136-infected hamsters relative to DD503-infected hamsters, plotted using the Kaplan–Meier method (Mantel–Haenszel log rank test; $P = 0.0133$; Fig. 4A). In the BALB/c mice inhalation model of

**Fig. 4.** *lfpA* is required for optimal virulence *in vivo*.

A. Virulence in Syrian hamsters. A targeted dose of 17 and 21 cfu of DD503 and RU136, respectively, were delivered to groups of five hamsters and survival was monitored for 6 days. Survival data were plotted using the Kaplan–Meier method and, using the Mantel–Haenszel log rank test, RU136 is significantly less virulent than DD503 ($P = 0.0133$).

B. Virulence in BALB/c mice. Approximately 2 LD₅₀ of DD503 and RU136 were delivered to groups of 10 mice and animal survival was monitored for 8 days. Survival data were plotted using the Kaplan–Meier method. RU136 is significantly less virulent than DD503 ($P = 0.0001$, using the Mantel–Haenszel log rank test).

Fig. 5. Prokaryotic and eukaryotic homologues of LfpA are highly conserved but phylogenetically distinct.

A. CLUSTAL W alignment of LfpA homologues from diverse prokaryotic and eukaryotic species indicating high levels of conservation across lineages. Amino acid sequence identity is indicated in bold type and dark grey shading. Sequence similarity is indicated in light grey shading. Accession numbers are provided in Table 1 or are as follows: *Mycobacterium smegmatis*, incomplete genome contig 3563; *Salmonella typhi*, NP_806334; *Streptomyces coelicolor*, NP_626682.

B. Phylogenetic analyses. The multispecies rectangular cladogram was generated by the unweighted pair group method with arithmetic mean (UPGMA) using random tie breaking and uncorrected pairwise distances in MacVector 7.0 (Oxford Molecular). Numbers on branches denote the UPGMA bootstrap percentage using a highly stringent number (1000) of replications (Felsenstein, 1985). All bootstrap values are $\geq 50\%$, as shown. Eukaryotic organisms are shaded. Accession numbers for many proteins in the tree are provided in Table 1 or in A. Others are as follows: *Bacillus cereus*, AAU17919; *T. volcanium*, NP_111448; *S. tokodaii*, BAB67667; *Drosophila melanogaster*, AAN09306; *Agrobacterium tumefaciens*, NP_396398; *Cryptococcus neoformans*, AAW44932; *Xanthomonas campestris*, NP_637112; *Caulobacter crescentus*, NP_420613.

acute melioidosis (Jeddeloh *et al.*, 2003), the LD₅₀ for strain RU136 was 14 927 cfu, in contrast to < 3000 cfu for DD503, representing a > 5.0-fold increase in LD₅₀. Furthermore, median survival was 40% greater for the RU136-infected mice relative to DD503-infected mice, plotted using Kaplan–Meier method (Mantel–Haenszel log rank test; $P = 0.0001$; Fig. 4B). The data from the two animal models of infection indicate that *lfpA* is required for optimal virulence, as a significant increase in LD₅₀ and time to death was observed for the *lfpA*-mutant strain in both models.

LfpA orthologues are widespread in prokaryotes and eukaryotes and are well conserved

Using the predicted amino acid sequence of LfpA as a search term, over 35 prokaryotic orthologues were identified in genome sequences of diverse bacteria including plant and mammalian pathogens, environmental organisms and extremophiles. LfpA orthologues were also detected in 13 eukaryotic species. Multiple alignments of prokaryotic and eukaryotic orthologues using CLUSTAL W (Thompson *et al.*, 1994) indicate that many residues are completely conserved across both prokaryotes and eukaryotes (Fig. 5A). Phylogenetic analysis indicates that prokaryotic and eukaryotic LfpA orthologues may be grouped according to lineage (Fig. 5B). Eukaryotic orthologues of LfpA derive from a single node indicating a common ancestor that is distinct from that of the prokaryotes (Fig. 5B). Furthermore, prokaryotic LfpA orthologues form two groups: members of the extremophiles (*Thermoplasma volcanium* and *Sulfolobus tokodaii*) and the genus *Bacillus* have a separate ancestry compared with the other prokaryotic proteins included in the analysis (Fig. 5B).

Discussion

Natural infection with *B. pseudomallei* may lead to the formation of multinucleate cells, and MNGC formation can be replicated *in vitro* (Harley *et al.*, 1998b). Cellular fusion following *B. pseudomallei* infection may represent a host response to attempt to contain the organism (as in the

formation of granulomas) or may represent a bacterially stimulated phenomenon providing the bacteria with a niche in which they may replicate and from which they may spread. Two *B. pseudomallei* gene products have been identified that play a role in MNGC formation (a type III secreted effector protein, BipB and the global regulatory factor RpoS) (Suparak *et al.*, 2005; Utaisincharoen *et al.*, 2006), suggesting that MNGC formation following *B. pseudomallei* infection is bacterially induced.

Multinucleated giant cells are abundant in biology (Vignery, 2000). They include Langhans giant cells, foreign-body giant cells (FBGCs), osteoclast-like cells and osteoclasts, and can be found in granulomas, FBGC reactions, giant cell tumours and bone respectively. To date, no studies have attempted to categorize *B. pseudomallei*-induced MNGCs.

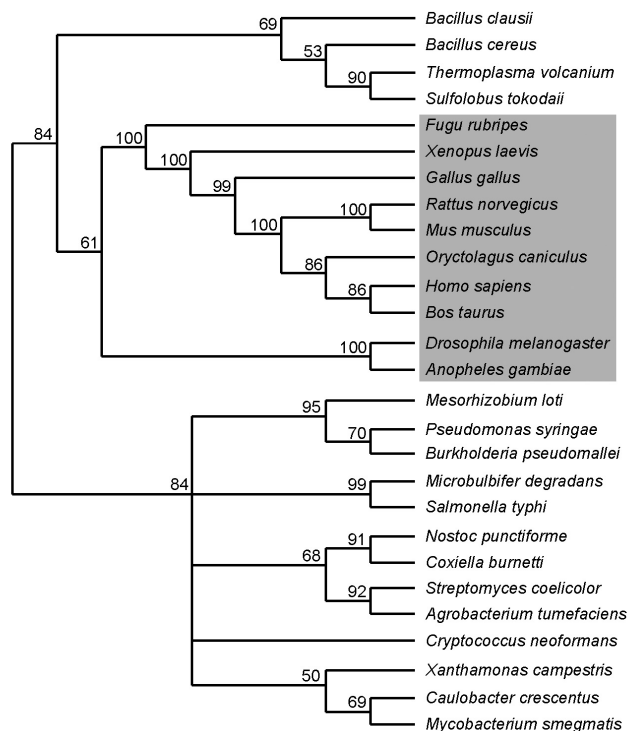
Osteoclasts are bone-resorbing cells that, like macrophages and dendritic cells, are derived from monocyte precursors. A number of reports indicate that *B. pseudomallei* infection can lead to chronic bone infections including osteomyelitis, where surgical debridement, extensive antibiotic treatment and careful long-term follow-up are required to reduce recrudescence (Subhadrabandhu *et al.*, 1995). In addition, melioidotic arthritis may account for up to a third of all septic arthritis cases in melioidosis-endemic regions (Kosuwon *et al.*, 2003). It may be that bone involvement is overlooked when other more obvious foci of infection are present, as one study indicates that the bacteria may be cultured from bone marrow samples almost as frequently as from blood cultures in patients with severe, acute melioidosis (Dance *et al.*, 1990). Also, in an acute infection model using SWISS mice, *B. pseudomallei* was isolated from bone marrow (Gauthier *et al.*, 2001). Thus the musculoskeletal system may represent a primary or secondary node of infection.

This study was undertaken to determine whether cells from *B. pseudomallei*-infected cultures resemble osteoclasts, by analysing expression of chemokines, a transcription factor and effector proteins characteristic of osteoclasts, and comparing with expression in model osteoclasts. The results demonstrate that following *B. pseudomallei* infection, MNGCs are observed after

A

<i>Homo sapiens</i>	MSSIKTECVLPENCRCGESPVWSEVSNLLFVDIPAKKVCRRWDSFTKQVQRVTMDAPVSSVALRQSG-GYVATIGTKFCA	10	20	30	40	50	60	70	80
<i>Rattus norvegicus</i>	MSSIKTECVLPENCRCGESPVWSEVSNLLFVDIPAKKVCRRWDSFTKQVQRVTMDAPVSSVALRQSG-GYVATIGTKFCA								
<i>Burkholderia pseudomallei</i>	MEHEFKWCAVPLASGCGESAVWAEAEQALYWDVTFRLLRLTPRDGCVKWTWLFDEPVVALSLTDRVGRLLVALGSHLIL								
<i>Coxiella burnetii</i>	MNIEIIVQGNALGEGPMWHYPYENVLYWIDIAKPSLHRLNPVTGGDYHQWKMPPDFIGAVVPRESG-GVITVGNVAF								
<i>Mycobacterium smegmatis</i>	MGTAINRDYSITVADLKTTLGEGPLWTEQCQLLYWLDISADGRIIPRATADGTQIRAWDVREKIGSMITLYRDGTAAALTALENGLYR								
<i>Salmonella typhi</i>	MTTTHLLFDYVGHLPCEPTWSEDESAIYWDILEQEIYHYHPASGTHSVLAHPEEVGSCFALREQG-GPIVAMRHAILWL								
<i>Streptomyces coelicolor</i>	MKRGTATAEVVAHHEATLGECEPTWSEDESAIYWDILEQEIYHYHPASGTHSVLAHPEEVGSCFALREQG-GPIVAMRHAILWL								
	GE.P.W	L	D	..	NSR	R	THDPA	TGRRTVRRTDQHVGA	..
<i>Homo sapiens</i>	LNNWKEQSAVVLAATVDNDKKNRFRNDGKVDPAGRYFAGTMAEETAPAVLERHCGA---	90	100	110	120	130	140	150	160
<i>Rattus norvegicus</i>	LNNWKEQSAVVLAATVDNDKKNRFRNDGKVDPAGRYFAGTMAEETAPAVLERHCGA---								
<i>Burkholderia pseudomallei</i>	WTAAATDERVNYGVWLDGWQVRFNEGRSGPDGSFWVGSMRNNVNTDGSLEAGGSDGILMRVGGPGTCTSIQSGSGLGIA								
<i>Coxiella burnetii</i>	VQIFPSGKMTCAAIEPWSDELRMNDGKCDRQGRFWIGVIANLDT-----ENPKGG---								
<i>Mycobacterium smegmatis</i>	VDIETGEQTEVLELERDLPSNRLNDGKVDKRRGRFVFGSMDTLE-----ESRSK---								
<i>Salmonella typhi</i>	ADKNGLLGRKVCNDPNSNTKLARFRNDGKVDGDDGRFYAGTFWAPG-----DYNGAL---								
<i>Streptomyces coelicolor</i>	LDEDD--TFRWLRREP-VPGRRGNDAAVAPGSLWAGTMRYDE-----ARGGG---								
	R..NDG	G	..G..	..	G	L	..
<i>Homo sapiens</i>	DHKIFXYIDSLSYSDAFDYDLQTDQIISNRRSVYKLEKEEQ-I	180	190	200	210	220	230	240	250
<i>Rattus norvegicus</i>	DHKIFXYIDSLSYSDAFDYDLQTDQIISNRRSVYKLEKEEQ-I								
<i>Burkholderia pseudomallei</i>	DHSRLIFGDTLANMLYIADYRDG--VLNNRHPEFTGFERGL--								
<i>Coxiella burnetii</i>	DNKIFXYIDSLSYSDAFDYDLQTDQIISNRRSVYKLEKEEQ-I								
<i>Mycobacterium smegmatis</i>	DGRTLYCDTWGTEWICYDYDLDTGKVSNNRPFARVDTSGGGAADGATVDA								
<i>Salmonella typhi</i>	DNQWMTSDTFTNGLIYRTLLDKH--GEPGKRRLEHFGEGEG-L								
<i>Streptomyces coelicolor</i>	DGTLMTADTFTRRRVDVTDADG--GRVAGRRPLAAIEDGAG-F								
	D	..Y	D	..	G	..R	..	PDG	..D
<i>Homo sapiens</i>	TTS CC FGGKNYSEMYVTCARDGMDEGLLRQPEAGGIFKITGLGVKGIAPYSYAG	260	270	280	290	300	310	320	330
<i>Rattus norvegicus</i>	TTS CC FGGKNYSEMYVTCARDGMDEGLLRQPEAGGIFKITGLGVKGIAPYSYAG								
<i>Burkholderia pseudomallei</i>	PTTCAFGGPDYRTLVTSAIHT-----PGDRLAGGVFAIR-TEVPGLPSFAFY								
<i>Coxiella burnetii</i>	PTSCIFGGANLDRLYITSCSQGTG-EAETLPPPAAGVFAIN-VGVRGLPEPAFVG								
<i>Mycobacterium smegmatis</i>	PTSMVFGGPDLDLVYTSMARPPPLFRFPEDGQLRGSLLFAIHGLGVGVHPRFGA								
<i>Salmonella typhi</i>	PTMVCFGGADMTLFTTTTRENMAQEVADYPLSGAIFTLQ-VAAGMKKSRFIERQAGSTGTTFSLG								
<i>Streptomyces coelicolor</i>	VTAACAFGGPDLDLTLYITTTARVGNP-----DPHPLAGSLLVIVP-DAGRGVAPAFAG								
	T	FGG	..Y..T	..	P	---	DPHPL	G	..F

B



12 h, as previously reported, and expression of the chemokines MCP-1, MIP-1 γ and RANTES is increased, as is NFATc1 expression. Recent studies have identified the importance of MCP-1, MIP-1 γ , RANTES and NFATc1 in osteoclastogenesis with essential roles in cell motility, attraction and multinucleation (Kyriakides *et al.*, 2004). Expression levels of the chemokines MCP-1 and MIP-1 γ , and transcription factor NFATc1 were higher in

B. pseudomallei-infected cells than in osteoclasts generated by treatment of RAW264.7 cells for 5 days with RANKL. As infected cells undergo apoptosis prior to this time, we compared expression of the chemokines and NFATc1 in RAW264.7 cells treated with RANKL for only 12 h, i.e. early in the differentiation pathway. Remarkably, gene expression by infected cells strongly resembles that of 12 h RANKL-treated cells, suggesting that

B. pseudomallei infection may drive RAW264.7 cells towards an osteoclast phenotype. Infection with *B. thailandensis* also resulted in increased expression of MCP-1, MIP-1 γ , RANTES and NFATc1 relative to uninfected cells, and also resulted in MNGC formation. Intriguingly, MNGCs were rarely observed following 12 h RANKL treatment of cells, despite expressing similar levels of these key osteoclastogenic factors. This implies that *Burkholderia*-infected cells express additional factors that mediate fusion. It remains to be seen what additional host cell responses lead to the rapid fusion and multinucleation of infected cells, when compared with cells exposed to RANKL.

We next examined expression of genes whose products are frequently used to define the osteoclast phenotype. CTR is a member of the seven-transmembrane G-protein coupled receptor superfamily (Pondel, 2000). Osteoclasts are a major target for calcitonin (Pondel, 2000), which, by means of its receptor, CTR, inhibits osteoclast-mediated bone resorption (Friedman and Raisz, 1965). CTSK and TRAP are expressed by bone marrow-derived osteoclasts, but not by bone marrow-derived dendritic cells or macrophages (Rho *et al.*, 2002). TRAP may be expressed by FBGCs (Kadoya *et al.*, 1994) and other non-osteoclastic MNGCs (Dersot *et al.*, 1995; Vignery, 2000). Alveolar macrophages also express TRAP, and this enzyme may have a role in generating bactericidal oxygen radicals (Bune *et al.*, 2001; Raisanen *et al.*, 2001; Raisanen *et al.*, 2005). TRAP is a key enzyme involved in bone degradation by active osteoclasts (Hayman *et al.*, 1996). CTSK is also expressed by osteoclasts, and is required for their full function (Gowen *et al.*, 1999). Expression of each of these marker genes was significantly increased by *B. pseudomallei*-infected cells compared with uninfected cells. When compared with 5 day RANKL cells, however, levels of CTSK and TRAP were significantly lower, suggesting that the infection may not result in functional osteoclasts.

Calcitonin receptor levels were strikingly higher in *B. pseudomallei* strain 08-infected cells compared with 12 h or 5 day RANKL-treated cells. This high-level expression of CTR is also observed following MCP-1 treatment of human monocytes, which results in TRAP-positive, CTR-positive MNGCs that are incapable of bone resorption (Kim *et al.*, 2006). The high-level expression of CTR following bacterial infection is, to our knowledge, previously unreported.

Burkholderia thailandensis-infected cells were morphologically indistinguishable from *B. pseudomallei*-infected cells. Expression of the chemokines MCP-1, MIP-1 γ , RANTES and NFATc1 were markedly induced by both species relative to uninfected cells. It is therefore remarkable that *B. thailandensis*-infected cells do not induce expression of the osteoclast markers CTR, CTSK and

TRAP. Thus the host cell response to these two organisms is markedly different, and the *B. thailandensis*-induced response does not resemble an osteoclastogenic pathway. In terms of induction of the osteoclast markers, *B. pseudomallei* > *B. pseudomallei* Δ *lfpA* > *B. thailandensis* = uninfected. It can be suggested, either that *B. pseudomallei* strains express additional factors that induce expression of these markers, or that *B. thailandensis* directly suppresses expression of these markers.

We identified a gene present in *B. pseudomallei*, absent from *B. thailandensis* by subtractive hybridization (Brown and Beacham, 2000). Mutation of the gene, *lfpA*, did not affect growth rate *in vitro*, nor did mutation affect bacterial intracellular proliferation. Our results, however, indicate an important role for *lfpA* in the interactions between *B. pseudomallei* and RAW264.7 cells. *lfpA* is itself induced when bacteria are in association with RAW264.7 cells. Expression of all chemokines (except RANTES) and all osteoclast markers was significantly reduced after infection with the Δ *lfpA* strain, JAB136, compared with the parental strain; complementation of the mutation restored the host cell response. However, cells infected with the Δ *lfpA*-strain JAB136 still expressed higher levels of the osteoclast markers CTR, CTSK and TRAP than did cells infected with *B. thailandensis*, implying that *lfpA* is not the only mediator of development of the osteoclast-like phenotype following infection.

The closely related pathogen *B. mallei* also induces formation of MNGCs after infection of RAW264.7 cells (Harley *et al.*, 1998b). Like *B. thailandensis*, *B. mallei* lacks the genomic island that contains *lfpA* (Holden *et al.*, 2004). It is not known whether the absence of *lfpA* from *B. mallei* leads to MNGCs that resemble the osteoclast-like *B. pseudomallei* MNGCs, or the *B. thailandensis*-induced MNGCs lacking CTR, TRAP and CTSK.

The *B. pseudomallei*-induced MNGCs were unable to form authentic resorption pits on dentine. This may be a consequence of insufficient CTSK production, as increased levels in *B. pseudomallei*-infected cells were only approximately 3% of that produced by cells treated for 5 days with RANKL. Importantly, however, very high magnification (10 000 \times) of particular 'affected' areas revealed regions of dentine resembling matrix. Bone comprises two phases: an inorganic, primarily calcium-phosphate phase and an organic protein matrix phase, composed mostly of type I collagen. While an acidic microenvironment can degrade the inorganic phase of bone, proteases are required for the degradation of the exposed organic phase. The observation of demineralized dentine without excavation of the matrix is also seen in osteoclasts from CTSK knockout mice in which demineralization of bone by osteoclasts occurs normally, but inadequate removal of the bone matrix was observed (Gowen

et al., 1999). This suggests that, in a manner analogous to MCP-1 and M-CSF treatment of human precursor cells (Kim *et al.*, 2005), insufficient CTSK may be produced by *B. pseudomallei*-infected RAW264.7 cells to permit efficient excavation of dentine under the tested conditions. The deficit in TRAP expression, relative to 5 day RANKL-treated cells, may also be associated with the inability of *B. pseudomallei*-infected RAW264.7 cells to effectively resorb dentine.

Another requirement for bone resorption by osteoclasts is the containment of extracellular enzymes by the formation of tight adherence to bone surface, mediated by integrins (Duong and Rodan, 1998). Genuine osteoclasts also demonstrate a peripheral actin ring (reviewed in Lakkakorpi and Vaananen, 1996). *B. pseudomallei* can induce polymerization of host-cell actin, which is regarded as a mechanism for intercellular spread (Kespichayawattana *et al.*, 2000), mediated by the BimA protein (Stevens *et al.*, 2005a,b). The formation of 'actin tails' behind bacteria presumably has a major effect on the host cytoskeleton, which may consequently interfere with the resorptive ability of such cells. This phenomenon, in addition to the low expression of CTSK and TRAP, relative to 5 day RANKL-treated cells, may explain the absence of authentic bone resorption by *B. pseudomallei*-infected RAW264.7 cells. However, this does not invalidate our hypothesis that *B. pseudomallei* induces osteoclast formation *in vivo*: if the bacteria infect and induce multinucleation in a monocytic precursor, the production of chemokines and differentiation signals may affect other, non-infected, local monocyte precursors, and drive their differentiation into a non-macrophage phenotype.

It is of note that SMP-30/regucalcin of rat, a eukaryotic orthologue of LfpA, stimulates the production of osteoclast-like cells in bone marrow cultures in a dose-dependent manner; calcitonin reduced the regucalcin-induced osteoclast-like cell formation (Yamaguchi and Uchiyama, 2005). In rats overexpressing regucalcin, increased osteoclastic activity leads to a reduction in bone mass as age increases (Yamaguchi *et al.*, 2004). Whether LfpA has a direct effect on the monocyte precursor cells, or is directing expression of other bacterial factors that lead to the osteoclast-like phenotype following *B. pseudomallei* infection awaits elucidation.

Bacterially induced osteoclastogenesis is not without precedent (see review by Nair *et al.*, 1996). Multinucleation and TRAP production by spleen cells has been reported following treatment with *Porphyromonas gingivalis*, a periodontal pathogen, or lipopolysaccharide from *E. coli* (Abu-Amer *et al.*, 1997; Jiang *et al.*, 2002), and the addition of surface-associated material from *P. gingivalis*, *Actinobacillus actinomycetemcomitans*, or *Staphylococcus aureus* to bone marrow cultures reportedly causes multinucleation and bone resorption *in vitro* (Wilson *et al.*,

1986; Wilson *et al.*, 1993; Nair *et al.*, 1995), although this effect may be due to an increase in RANKL by associated osteoblasts, rather than a direct effect on osteoclast precursors. However, incubation periods of 10 days or more were routine in these studies. The potent modifications induced by *B. pseudomallei*-infected cells observed in this study occur after only 12 h, implying a rapid host cell response. Whether this has pathological significance in human disease, for example in chronic bone disease (see earlier) or in the provision of a niche in which *B. pseudomallei* may replicate, is unclear. However, our *in vivo* studies indicate that mutation of *lfpA* (which in part modulates osteoclast marker expression) results in attenuation in two animal models of acute infection. It remains to be seen what effect this gene may play in animal models that more closely resemble the chronic disease state such as C57Bl/6 mice (Leakey *et al.*, 1998).

Further analysis of *B. pseudomallei*- and *B. thailandensis*-infected cells and comparison with 12 h-RANKL-treated cells may yield not only insights into host response to infection, but also information fundamental to understanding cellular fusion events; investigations of the molecular events following *B. thailandensis*-, *B. pseudomallei*- and *B. mallei*-infection will provide an advance in understanding host responses to pathogenic and non-pathogenic bacteria. Furthermore, experiments with other bacterial species will determine whether the novel observation that CTR expression is raised following *B. pseudomallei*-infection is a specific response, or a general response to bacterial infection. A detailed analysis of the function of LfpA will add significantly to our understanding of the pathogenesis of an agent with potential threat as a bioweapon. Finally, further analyses will determine whether the osteoclast-like phenotype of *B. pseudomallei*-infected cells represents a novel mechanism for subversion of the immune system.

Experimental procedures

Bacterial strains, plasmids and growth conditions

The bacterial strains and plasmids used in this study are described in Table 2. Bacteria were grown at 37°C on LB agar or in LB broth shaken at 200 r.p.m. overnight (250 r.p.m. for animal infection studies). Antibiotics were added to the following final concentrations where appropriate: ampicillin, 100 µg ml⁻¹; chloramphenicol, 50 µg ml⁻¹ (*E. coli*) and 100 µg ml⁻¹ (*B. pseudomallei*); gentamicin, 10 µg ml⁻¹ (insertion mutagenesis) and 15 µg ml⁻¹ (remainder); streptomycin, 100 µg ml⁻¹; tetracycline, 10 µg ml⁻¹, imipenem, 10 µg ml⁻¹.

In silico sequence analyses

The *B. pseudomallei* genome sequence was obtained from the Sanger Research Institute (<http://www.sanger.ac.uk/>). DNA

Table 4. Oligonucleotides used in this study.

Oligonucleotide	5' to 3' sequence	Enzyme ^a	Reference
Cloning/mutagenesis			
136USF	<u>gctctagacaaactgctccttaggtcacgc</u>	XbaI	This study
136USR	<u>cgggatcctacaacgcgtgtccgcctcg</u>	BamHI	This study
136DSF	<u>cgggatccatccgccagcatccatactgag</u>	BamHI	This study
136DSR	<u>ggggtagcccgccatcgctgtgagagagtg</u>	KpnI	This study
RgcUSF	<u>ccttcagctcagattgaaagag</u>	None	This study
RgcDSR	<u>ggagcaggtgaatctcttcaa</u>	None	This study
RgcExpF	<u>gctctagaccttcagctctgattgaaagag</u>	XbaI	This study
RgcExpR	<u>gctctagaggagcaggtcaatctcttcaa</u>	XbaI	This study
RU136F	<u>aacatgaattcaagtgggtctgc</u>	None	This study
RU136R	<u>agtaggatgctggcggatgt</u>	None	This study
Q-PCR analysis			
RgcRT F (<i>lfpA</i>)	caggcgtgtactggacaga	None	This study
RgcRT R (<i>lfpA</i>)	tcggcgatgtagagcatgt	None	This study
16S 325–345	agacacggcccagactctac	None	Brett <i>et al.</i> (1997)
16S 625–645	cagtcaccaatgcagttccca	None	Brett <i>et al.</i> (1997)
CTR F	gcgtgctgtgaccaagatg	None	This study
CTR R	gaggagcactaactacgcgg	None	This study
CTSK F	tgtataacgccacggcaaagg	None	This study
CTSK R	catggttcacattatcacgggtca	None	This study
MIP-1 γ F	cagagcagctgaaggcacag	None	This study
MIP-1 γ R	cgtagttataggacaggcagc	None	This study
NFATc1 F	tctcaaggaacgagaagggct	None	This study
NFATc1 R	atatgcctggtgtggtcaga	None	This study
RANTES F	catgaagatctctgcagctgcc	None	This study
RANTES R	gcacttgctgctggtgtaga	None	This study
TRAP F	gcctacctgtgtggacatga	None	This study
TRAP R	gaccttctgtgtgtcgcac	None	This study
18S F	cttagagggacaagtggcg	None	This study
18S R	acgctgagccagtcagtgtga	None	This study

a. The listed endonuclease recognizes the underlined sequence of the oligonucleotide and cuts within it.

sequences, reading frames and protein sequences were analysed using Artemis Release 4 (Rutherford *et al.*, 2000). Nucleotide and protein comparisons were conducted using the suite of programs hosted by the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>) and included various BLAST (Altschul *et al.*, 1997) searches. Additional motif analyses were made using the ScanProsite database (Gattiker *et al.*, 2002), available at <http://www.expasy.org/tools/scanprosite/>. Signal peptide analyses were undertaken using SIGNALP 3.0 (<http://www.cbs.dtu.dk/services/SignalP/>) with both neural networks and hidden Markov models trained on Gram-negative bacteria (Bendtsen *et al.*, 2004). Restriction profiling, oligonucleotide primer compatibility, CLUSTAL W alignments (Thompson *et al.*, 1994) and phylogenetic analyses were performed using MacVector 7.0 (Oxford Molecular).

Mutagenesis and complementation

Two types of mutagenesis were employed in this study: unmarked in frame deletion mutagenesis and insertional inactivation mutagenesis. The unmarked in frame $\Delta lfpA$ deletion mutant was generated in *B. pseudomallei* 08 by allelic exchange using the methods described previously (Blomfield *et al.*, 1991; Brown *et al.*, 2004; Essex-Lopresti *et al.*, 2005). The $\Delta lfpA$ allele was generated by polymerase chain reaction (PCR) using the oligonucleotide primer pairs 136USF/USR (61°C annealing) and 136DSF/DSR (61°C annealing; see Table 4). PCR amplicons were digested with BamHI, ligated, and, owing to the presence in

strain 08 of a KpnI site in the DSF/R PCR product that was absent from the genome sequence of strain K96243, the 1764 bp fragment (representing the $\Delta lfpA$ allele) was subcloned into pGEM-T Easy, generating pJAB136easy. The $\Delta lfpA$ allele was excised from pJAB136easy with SpeI and SphI and cloned into pDM4, generating the deletion construct pJAB136. This was conjugatively mobilized from *E. coli* S17.1 (λ pir) into *B. pseudomallei* 08 and integration of pJAB136 by homologous recombination, involving the upstream *lfpA* portion, was selected for with chloramphenicol and confirmed by PCR (RgcUSF/DSR primers; 59°C annealing; see Table 4) and Southern blot analyses (results not shown). Excision of pJAB136 by a second homologous recombination event, involving the downstream *lfpA* portion, was selected on LB agar containing 10% sucrose, but lacking NaCl (incubated at 30°C for 2–3 days), and chloramphenicol sensitivity, and was confirmed by PCR, Southern blot analyses and Q-PCR of *lfpA* mRNA expression (results not shown). The unmarked and in frame $\Delta lfpA$ strain was designated JAB136. Growth of JAB136 on LB agar or in liquid media was not impaired relative to the parental strain.

The insertional inactivation of *lfpA* was achieved in *B. pseudomallei* DD503 (Ulrich *et al.*, 2004). Briefly, an 803 bp PCR amplicon internal to *lfpA* was amplified from DD503 DNA using primers RU136F/R (60°C annealing; see Table 4). The amplicon was subcloned into pCR2.1-TOPO, digested with EcoRI, and cloned into the suicide vector pGSV3 (DeShazer *et al.*, 2001), creating pRU136. This integration cassette was mobilized into DD503 by conjugation, merodiploids selected on gentamicin, and site-specific integration was confirmed by long-

range PCR (results not shown). The *lfpA*::pGSV3 strain was designated RU136.

The complementation construct, pJAB136C, was generated by PCR amplification of *lfpA* and upstream DNA using DNA *pfu* Taq polymerase (Invitrogen) with primers RgcExpF/R (57°C annealing; see Table 4) and cloning the amplicon into pGEM-T Easy, generating pJAB136Ceasy. The cloned fragment was excised with XbaI and ligated into pBBR1MCS-3, generating pJAB136C. The complementation construct was conjugatively mobilized into JAB136 and selected with tetracycline, generating strain JAB136C. Complementation *in trans* was confirmed by PCR of plasmid DNA extracted from JAB136C, and by Q-PCR of *lfpA* mRNA expression in contact with RAW264.7 cells.

Cell maintenance and infection assays

RAW264.7 cells were grown in minimal essential medium (MEM) with Earl's salts and L-glutamine (Gibco), supplemented with 10% (v/v) heat-inactivated fetal calf serum (FCS; Gibco), at 37°C, in 5% CO₂. During maintenance, this medium also contained 1× penicillin/streptomycin solution (Gibco). Cells were subcultured three times weekly at a ratio of 1:8 using a 22-gauge Neolus needle and 5 ml Neolus syringe (Terumo Corporation).

RAW264.7 cells were seeded to a density of 5.0×10^4 cells per 2.0 cm² growth area. The following day, maintenance medium was replaced with antibiotic-free medium and cells were infected with bacteria grown for 16 h at a multiplicity of infection (moi) 10 bacteria per cell and incubated at 37°C, 5% CO₂. For Q-PCR analysis of *lfpA* mRNA expression, bacteria were inoculated into T-25 flasks (Greiner BioOne) containing or lacking RAW264.7 cells, and incubated for 3 h. Bacteria were recovered from the culture lacking RAW264.7 cells by centrifugation, and resuspended in 4 M guanidium isothiocyanate, 1% lauryl sarcosine. Infected RAW264.7 cells were washed and lysed with 4 M guanidium isothiocyanate, 1% lauryl sarcosine.

For Q-PCR analysis of RAW264.7 cells treated with either *B. pseudomallei*, *B. thailandensis* or RANKL (5 ng ml⁻¹; Peprotech), T-75 flasks (Greiner BioOne) were used and incubation time was 12 h and 5 days respectively. The culture medium was removed; cells were washed twice with phosphate buffered saline (PBS) and resuspended in 4 M guanidium isothiocyanate, 1% lauryl sarcosine.

Intracellular proliferation assays

Intracellular proliferation assays were performed according to previously described methods (Brown *et al.*, 2004). In this study 5.0×10^4 RAW264.7 cells were seeded into 24-well tissue culture plates (Greiner Bio-One) in antibiotic-free medium and incubated overnight to allow attachment. Cells were infected at moi 10 bacteria per cell and incubated at 37°C, 5% CO₂, for 1 h, to allow bacterial entry into cells. Culture medium was removed and replaced with medium containing 10 µg ml⁻¹ imipenem, to kill extracellular bacteria, and incubation continued. Intracellular bacteria were enumerated 4 h and 12 h post infection by viable count. This involved washing each well three times with culture medium to remove extracellular bacteria, permeabilizing cells with 0.1% Triton X-100 for 10 min at room temperature, and plating serial 10-fold dilutions onto agar. Colonies were counted after overnight growth at 37°C. Fold-proliferation was determined

by dividing the number of intracellular bacteria 12 h post infection by the number of intracellular bacteria 4 h post infection. Bacterial strains were investigated in triplicate wells per assay over triplicate assays.

Immunofluorescence imaging

Bacteria were inoculated at moi 10 onto RAW264.7 cells cultured on glass coverslips in 24-well plates, incubated for 2 h, washed, and media containing imipenem (10 µg ml⁻¹) added. After a further 12 h incubation, cells were washed with PBS, and fixed with 3.7% formaldehyde. After washing with PBS, cells were permeabilized with 0.1% Triton-x 100 in PBS for 10 min, and blocked with 4% FCS in PBS. Bacteria were labelled with rabbit polyclonal antibodies raised against the sarkosyl-insoluble fraction (enriched for outer-membrane proteins) of *B. pseudomallei* strain 08 and goat anti-rabbit alexaflor 488 as the secondary antibody. Actin was labelled with a rhodamine-phalloidin conjugate, and nuclei were labelled with DAPI. The coverslips were mounted on a microscope slide in 10% n-propylgalate to prevent bleaching, and sealed with nail polish. Images were recorded in monochrome using Nikon Eclipse E600 microscope and a Nikon CoolPIX 950 digital camera at three wavelengths. Images were false coloured (red for actin, green for bacteria, blue for DAPI) and assembled using ImageJ software.

RNA and cDNA preparation and Q-PCR analyses

Following cell lysis with 4 M guanidium isothiocyanate and 1% lauryl sarcosine, total RNA and first-strand cDNA was prepared as described previously (Day *et al.*, 2004). Q-PCRs were performed using the Bio-Rad iCycler real-time PCR platform. For murine gene analysis PCRs were optimized for an annealing temperature of 55°C, ~50 ng of cDNA and a primer concentration of 100 nM in a final volume of 20 µl in 1× Bio-Rad SYBR Green I master mix. The reactions were cycled as follows: step 1, 94°C for 2 min; step 2, 94°C for 30 s, 55°C for 45 s, 72°C for 1 min (45 cycles); step 3, melt curve analysis from 55°C to 95°C in 0.5°C increments. 18S rRNA was used as the housekeeping gene for all eukaryotic cell analysis. All fold were calculated by $\text{Fold} = 2^{\Delta\Delta\text{Ct}}$ as per the study by Livak and Schmittgen (2001) where $\Delta\Delta\text{Ct}$ is equal to (gene treatment 1 minus 18S treatment 1) minus (gene treatment 2 minus 18S treatment 2). For bacterial gene expression PCR was optimized for 60°C annealing temperature and cycled as earlier with the altered annealing temperature. 16S rRNA was used as the housekeeping gene for bacterial analysis. The cycle threshold (Ct) was calculated at 50% maximal fluorescence output for all genes and all samples. Standard curves were constructed of all primer sets by analysing incremental 10-fold increases in concentration of target DNA from 10 to 10⁹ copies of PCR product. Copy number was calculated from the equation of the line characterized for each primer set ($Y = MX + C$) where the Ct for a gene is Y, the number of cycles to increase the amount of product by 10 times is M, the log of the copy number is X, and C is the Y intercept where X = 0. The copy number is then solved and is divided by the copy number of 18S or 16S rRNA observed in a PCR (see Table 4). For bacterial gene expression PCR was optimized for 60°C and cycled as earlier with an annealing temperature of 60°C. 16S rRNA was used as the housekeeping gene for bacterial analysis.

In vitro bone resorption assay and microscopy

The dentine resorption assay was derived from protocols described previously (Hodge *et al.*, 2004; Kim *et al.*, 2005). In this study 96-well culture plates (Greiner BioOne) containing sterile dentine slices were seeded with 8.0×10^3 RAW264.7 cells and incubated at 37°C in 5% CO₂ overnight. Maintenance medium was replaced with antibiotic-free medium and cells were infected at moi 10 bacteria per cell and the mix was incubated at 37°C in 5% CO₂ for 24 h. Culture medium was replaced at 12 h. Wells were washed twice with PBS and samples fixed in 3.7% (v/v) formaldehyde in PBS for 30 min. Bacteria and cells were removed from the dentine surface using 3:2 methanol : chloroform. Dentine slices were mounted onto 9 mm stubs (ProSciTech) and gold sputter coated for 30 s at 25 mA in a SC500 sputter coater (Bio-Rad). Dentine slices were viewed using an FEI Quanta 200 Environmental Scanning Electron Microscope, operating at 10–20 kV.

Animal infection studies

The Syrian hamster model was derived from the methods used by Brett *et al.* (1997). Groups of five male Syrian gold hamsters were infected intraperitoneally with 10-fold dilutions (10^1 – 10^3) of bacteria grown at 37°C overnight at 250 r.p.m. The survival of hamsters was recorded for 6 days and LD₅₀ values were determined at day 3, as described by Reed and Muench (1938).

Infection of BALB/c mice by aerosolization occurred as previously described (Jeddeloh *et al.*, 2003; Ulrich *et al.*, 2004). Bacteria (100 µl from a 3 ml overnight culture) were inoculated into 10 ml of fresh LB broth and grown overnight at 37°C, 200 r.p.m. Groups of 10 mice were infected by nebulizing diluted aliquots of overnight culture, delivering approximately 2 LD₅₀, 4 LD₅₀, 6 LD₅₀ and 8 LD₅₀ (Jeddeloh *et al.*, 2003). LD₅₀ values were determined at day 5, as described by Reed and Muench (1938).

Statistical analyses

Statistical analyses of mRNA expression from Q-PCRs were undertaken using one-way ANOVA and appropriate *post hoc* tests using SPSS statistical package and intracellular proliferation assays were undertaken using the independent samples two-tailed *t*-test. Animal survival data were plotted using Kaplan–Meier method and statistically evaluated using the Mantel–Haenszel log rank test. All *t*-tests and animal survival data were performed in GraphPad Prism 4 for Windows.

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